FIGHT of the 21st CENTURY

★ REMATCH ★

TUBERCULOSIS VS. HUMANITY

EXPOSURE OF TB'S SECRET STRATEGY COULD SHIFT THE ODDS MORE IN OUR FAVOR.

Before antibiotics, public signs such as this one from England were a major component of tuberculosis control.

THE DISCOVERY OF STREPTOMYCIN in the mid-1940s turned the world on its ear. The broad application of this antibiotic drug abruptly reduced tuberculosis (TB) from the leading cause of death to a 20th century anachronism in the United States. Streptomycin and the arsenal of antibiotics discovered since are powerful tools in the fight against infectious disease. They, along with vaccines, are the best line of defense. But drug mismanagement, particularly over-prescription and misuse, can lead to resistance, reducing their ability to fight infection and actually strengthening the bacteria they were meant to kill. Tuberculosis is one of the diseases antibiotics have been helping the world beat, but it is showing worrisome trends in drug resistance.

In 1991, the World Health Organization (WHO) recognized the growing problem of TB and implemented a massive, globally coordinated effort to bring down the annual number of new infections and bring up the number of cured ones. Since that time, according to the latest WHO Global Tuberculosis Report, new cases are down by 41 percent and fatal outcomes have dropped by nearly half. So their strategy, which hinges on effective diagnosis, availability of drugs, and supervised adherence to treatment regiments, is working.

Still, over a million people die each year from TB. Roughly a third of the world's population is infected and nine million new infections occurred in 2013 alone. Don't know anyone with TB? That's because most infections occur in developing countries; in 2013, the highest incidences of fatal TB were in India, South Africa, Bangladesh, Pakistan, and Indonesia.

Tuberculosis is a contagious and airborne respiratory infection (though organs other than the lungs, such as the bones or lymph nodes, can be infected too). The causative agent, *Mycobacterium tuberculosis*, is a hearty species of bacteria that has plagued humans since time immemorial. Most infections are latent, meaning a person can be infected without developing the disease for a long time, sometimes throughout their life. However, about 10 percent of infected people develop the disease, or active TB, which is very dangerous and second only to human immunodeficiency virus (HIV) in the number of global deaths it causes each year. But TB and HIV aren't operating independently; in fact, they work in terrible synergy. First, HIV depresses the immune system, and then TB comes in as an opportunistic secondary infection and overwhelms the body's remaining defenses. Co-infection with HIV raises the likelihood of developing active TB by up to 20 times and accounts for a quarter of TB's death toll.

Co-infection with HIV is not the only thing driving TB's re-emergence, however. Recent decades have seen alarming increases in mortality due to new multidrug-resistant and extensively drug-resistant bacterial strains. Multidrug-resistant TB (MDR-TB) is defined as having resistance to the two most potent first-line drugs, isoniazid and rifampicin. Extensively drug-resistant TB (XDR-TB) is defined as having resistance to both of the first-line drugs, as well as other commonly used TB drugs. First identified during a highly lethal 2005 South African outbreak (98 percent dead within two weeks of diagnosis), XDR-TB has now been reported in 92 countries, including the United States. In recent years, there have been ominous, though rare and isolated, reports of totally drug-resistant TB strains that cannot be treated with any drugs at all.

Drug resistance is usually diagnosed *in vitro*—that is, in a culture dish in a lab—which can take weeks, is difficult to do in the field, and can have poor sensitivity and specificity. On-demand molecular diagnostic tests are becoming increasingly available, but many of the resource-limited, hardest-hit regions still use the culture method. In addition, culturing relies on a single sample, which may underrepresent the composition of an individual infection. So while determining the best treatment for a patient depends on a drug-resistance profile, such profiles can be inaccurate and difficult to generate. Improved outcomes rely on improved speed and accuracy of diagnosis, which might be achieved with improved data.

This is where Los Alamos theoretical biologist Bette Korber and her team come in. Korber works primarily with HIV, which led first to an interest in TB and next to an opportunity to study it. In collaboration with the National Institutes of Health (NIH) and the International Tuberculosis Research Center in South Korea, Korber and colleagues collected data on as many TB strains as they could get their hands on. They then analyzed the strains' genetic codes, transmission routes, and drug-resistance profiles, searching for ways that drug-resistance diagnosis might be improved.

SICKNESS, STATEHOOD, AND SITE Y

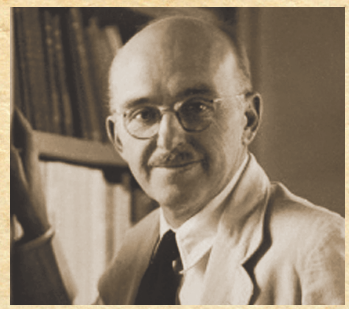
AT THE PEAK OF THE LUNGER MIGRATION, NEARLY A THIRD OF NEW MEXICO'S POPULATION HAD ARRIVED ON THE WINDS OF THE WHITE PLAGUE

At the turn of the 20th century, tuberculosis, also called consumption, was the leading cause of death in the United States. With no effective drugs, doctors prescribed fresh air, sunshine, rest, a hearty diet, and cultivation of a cheerful attitude. Consumptives wheezed their way to the American Southwest on doctors' orders or by their own accord, with hopes that the salubrious climate would cleanse their lungs and restore their vigor.

New Mexico received these medical refugees, colloquially called "lungers," by the thousands. While Texas, its neighbor to the east, maintained that consumptives were explicitly unwelcome, New Mexico eagerly built tuberculosis hospitals, or sanatoria, to accommodate the coughing droves. Military hospitals led the movement, but soon private sanatoria abounded, due largely to tax incentives codified in the 1903 "Act to Encourage the Establishment of Sanatoria in the Territory of New Mexico." By 1908, the lunger movement was in full swing and the territory's governor, jockeying for federal recognition, proclaimed New Mexico "the nation's sanatorium."

At the peak of the lunger migration, nearly a third of New Mexico's population had arrived on the winds of the white plague. In fact, the influx of health seekers and their comparatively high rates of recovery—New Mexico claimed the lowest tuberculosis-related death rate in the country—were cited by territory officials in support of their ongoing petition for statehood, which was granted in 1912.

But it's not just New Mexico's state history that was touched by tuberculosis. Los Alamos and its shadowy first ambition, the Manhattan Project, were also influenced by lungers in numerous and lasting ways.



Photograph used with the permission of the Amon Carter Museum of American Art

Architect **John Gaw Meem** came to New Mexico as an invalid in 1920, having contracted tuberculosis on the heels of the Spanish flu. As he convalesced at Santa Fe's Sunmount Sanatorium, he grew enamored with the Spanish Pueblo Revival style of architecture. After his recovery, he stayed in Santa Fe and dedicated his career to the conservation of New Mexico's architectural heritage. He became a preeminent purveyor of historically faithful architecture and designed dozens of iconic buildings throughout the state. Early in his career, in 1928, Meem was commissioned by Ashley Pond, the founder of the Los Alamos Ranch School, to build the school's central building, an imposing lodge of upright logs. The school was purchased in 1943 by the United States Army for use as "Site Y," the code name used to scout locations for the Manhattan Project's research laboratory. Meem's now-famous Fuller Lodge still stands and is adjacent to the Los Alamos Historical Museum.

Also in residence at Sunmount Sanatorium circa 1920 was Dorothy Scarritt, a tubercular recent college grad from Missouri. During her recuperation, Dorothy liked Santa Fe so much that years later—after having recovered, married, moved to the Midwest, and become suddenly widowed—she returned, now **Dorothy McKibbin**, to raise her child. When the top-secret Manhattan Project came to northern New Mexico, Dorothy was hired by J. Robert Oppenheimer, the project's civilian director. Her title was "secretary" but she was essentially the gatekeeper for all new arrivals to "the hill," as Los Alamos was called. While overseeing the flow of scientists, technicians, their families, and supplies up the hill, she diligently and skillfully preserved the project's secrecy—unaware herself of its true scope—which was vital to its ultimate success.

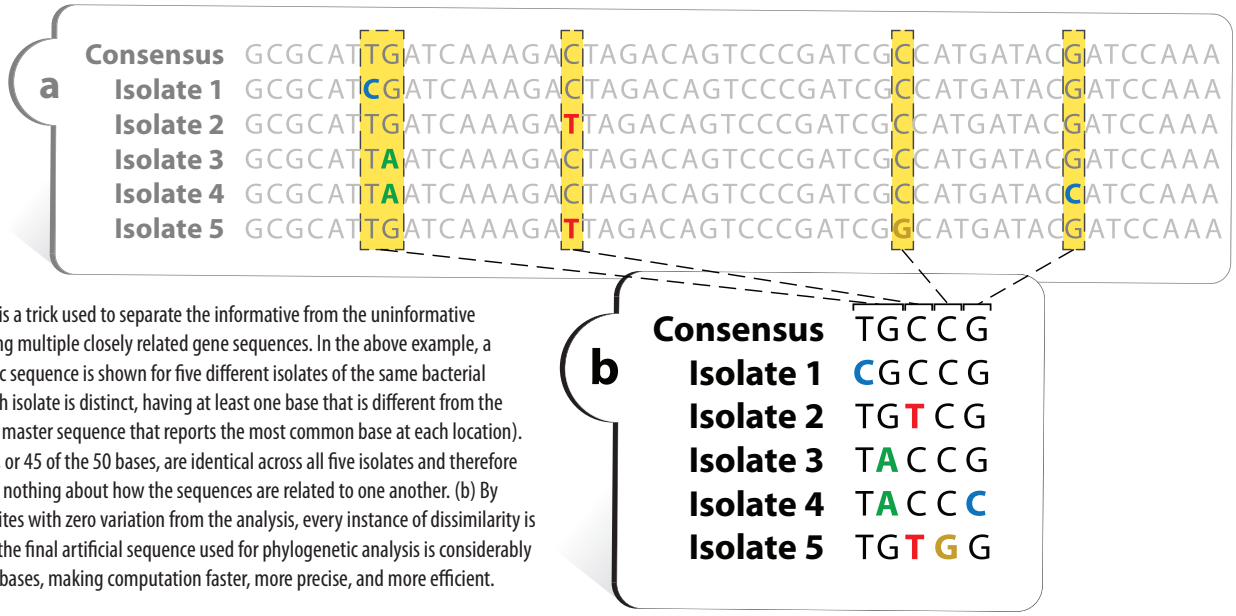
CREDIT: LANL archives

Photograph used with the permission of Melanie Jackson Agency, LLC

Tuberculosis nearly prevented the young **Richard Feynman**, famous physicist and Nobel laureate, from participating in the Manhattan Project. His wife, **Arline**, was terribly ill, and he would not leave her to come to Los Alamos. It was Oppenheimer who located a sanatorium in Albuquerque for her and who convinced Feynman that he could visit her on weekends while spending his workweek on the hill. Arline died from her illness in Albuquerque in June of 1945, just two months before the end of the war and nine months before streptomycin was proven as a cure.

CREDIT: LANL archives

Finally, **Oppenheimer** himself spent time recovering from tuberculosis in New Mexico. In 1922, just after graduating high school, he contracted severe dysentery while traveling in Europe and was sent to New Mexico with a chaperone to recuperate. Rather than snoozing in a chaise lounge, he traveled the hills by horseback and befriended local families. He so loved the culture, people, and landscape that when he was diagnosed with tuberculosis in 1928, he again came to northern New Mexico to recover. These health-seeking stints and subsequent visits were how he came to know the Los Alamos Ranch School and the Pajarito Plateau upon which it sat. So when General Leslie Groves asked if he had any thoughts on a good location for Site Y, Oppenheimer knew just the place.



Concatenation is a trick used to separate the informative from the uninformative when comparing multiple closely related gene sequences. In the above example, a 50-base genetic sequence is shown for five different isolates of the same bacterial species. (a) Each isolate is distinct, having at least one base that is different from the consensus (the master sequence that reports the most common base at each location). Ninety percent, or 45 of the 50 bases, are identical across all five isolates and therefore tell researchers nothing about how the sequences are related to one another. (b) By removing the sites with zero variation from the analysis, every instance of dissimilarity is preserved and the final artificial sequence used for phylogenetic analysis is considerably smaller, only 5 bases, making computation faster, more precise, and more efficient.

Minimizing here, maximizing there

M. tuberculosis comes in different classification types that are associated with particular geographic regions and clinical profiles. Determining which type a sample belongs to is done by looking for specific genetic markers that are diagnostic for that type. There are a few different kinds of tests that can be done, but they are limited in that they only look at part of the genome. Like the parable of the blind men trying to describe an elephant from touching only one part, important information is lost by not considering the whole. The Los Alamos team looked at whole genomes and compared what they saw to other researchers' reports of trunks, tails, and tusks.

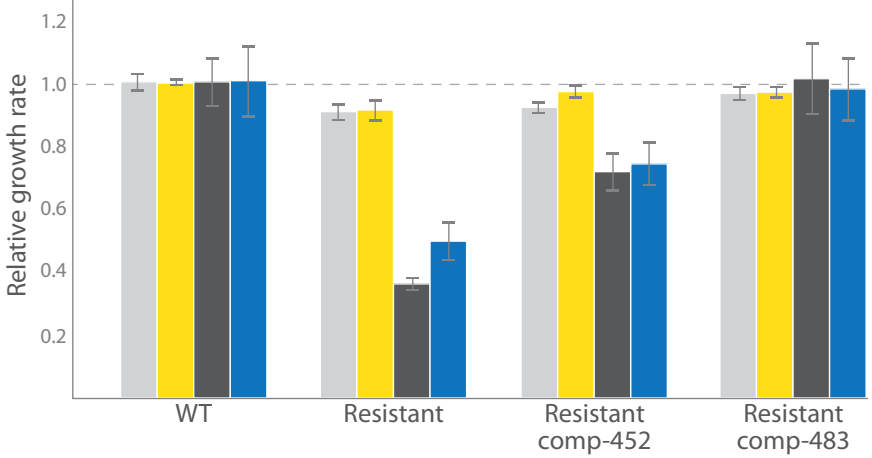
Viral immunologist Karina Yusim, a scientist in the group and expert in sequence analysis, wanted to use whole-genome sequence (WGS) data to put together a global evolutionary portrait of tuberculosis and map drug resistance within and between different outbreaks. The genome of *M. tuberculosis* is roughly four million bases long. (For comparison, influenza virus has 13.5 thousand bases and the human genome has 3.2 billion.) Comparing multiple strains on a genome level can be a bit tricky, so Shihai Feng, a programmer on Korber's team, wrote a computer code to reduce the amount of data without reducing its content by doing a data transformation called concatenation. Information about the origins and relatedness of genomes is gleaned from looking at the differences between genomes. Most of the four million bases are identical at each position across all strains (not surprising, given that all the strains were the same species, *M. tuberculosis*), but every so often one strain will have an A, for instance, where all the others have a G. This is a single nucleotide polymorphism, or SNP (pronounced "snip"). The SNPs are the variation, and the variation is they key, so by removing all the identical genome positions, each four-million-base genome was paired down to a more manageable size of roughly 18,000 variable bases.

That is still a relatively high number of SNPs. A handful of TB strains are very well studied, but because the Los Alamos researchers included all available WGSs, not just the well-studied ones, they discovered that some of the sequences had artifacts that looked like SNPs, but weren't. Feng, Yusim, and Korber developed a novel algorithm that would flag mutations suspected of being sequencing artifacts rather than SNPs of biological origin so they could omit those confirmed to be artifacts from further analysis.

Next they did a phylogenetic comparison of their concatenated WGS SNP data to the small subset of SNPs used by conventional methodology. Phylogenetic analysis compares numerous similar sequences and mathematically determines the most likely relationships between them to suggest patterns of evolution and transmission. They found that the inclusion of SNPs from the whole genome provided finer resolution of relationships between strains and could be important in figuring out how drug resistance evolves, which often involves just a single, well-placed SNP.

Greater than the sum of its parts

One of the most potent antibiotics against TB is the first-line drug rifampicin. It is also one to which TB becomes resistant most frequently. The TB gene *rpoB* (for RNA polymerase beta subunit) encodes the portion of RNA polymerase—an enzyme vital for bacterial reproduction—that rifampicin acts on. When rifampicin binds to a particular bulge on the enzyme's surface, it blocks a neighboring channel that contains newly synthesized RNA. If the RNA can't exit the channel, the enzyme becomes clogged and the



The cost of bacterial drug resistance and the restoration of fitness by compensatory mutation are most pronounced when resources are scarce—as would be the case in an ailing human body. Wild-type ("WT") *Mycobacterium smegmatis,* a close relative of *M. tuberculosis,* grows equally well in conditions of high sugar (white bars), low sugar (black bars), reduced carbon (yellow bars), and reduced carbon plus low sugar (blue bars). When a mutation conferring rifampicin resistance is introduced ("Resistant"), growth is significantly slowed for the low-sugar group and for the reduced-carbon plus low-sugar group. When either of two compensatory mutations are then added, either at position 452 ("Resistant comp-452") or at position 483 ("Resistant comp-483") within the *rpoC* gene, growth is either partially or completely restored, relative to the unaltered wild type.

bacteria can't grow. So if the bacteria want to prevent the action of the drug, one way to do that is to modify the shape of the particular bulge that rifampicin binds to so that it doesn't fit any more. And that's exactly what happens. Rifampicin resistance-conferring mutations all occur in a very narrow window in the middle of the *rpoB* gene, the part that encodes the bulge on the beta subunit bound by rifampicin. (This is not the case with other drugs.)

But resistance isn't free. It's been long understood that with acquisition of drug resistance there often comes a loss of fitness. The RNA polymerase enzyme's natural structure was optimized over eons of evolution, so to suddenly tweak its shape usually makes it less efficient and slows bacterial growth. However, the fitness defect is temporary while the drug resistance is not. Somehow, drug-resistant strains can regain fitness without losing resistance. Last year, it was finally revealed that the bacteria use a push-me-pull-you strategy, mutating a different part of the RNA polymerase to compensate for the shape change in *rpoB*.

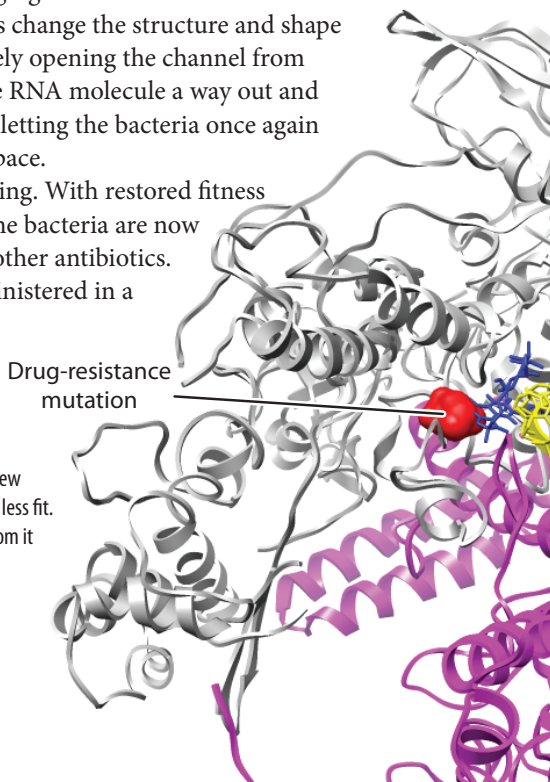
The Los Alamos team and its collaborators at NIH and in South Korea wanted to figure out just how these compensatory mutations worked. Korber and Yusim, together with Los Alamos physicist James Thieler (Korber and Theiler are not only colleagues, they happen to be married) looked at global TB WGS data as well as a set of data from highly antibiotic-treated patients in South Korea. When they compared patterns of mutation throughout the genome between drug-susceptible TB and drug-resistant TB, they found one gene stood out—it was the gene for a different part of the polymerase, called *rpoC*. Looking closely at *rpoC* gene sequences, they found that, although mutations in *rpoC* aren't required for rifampicin resistance, those mutations appeared most often in rifampicin-resistant strains and seemed to accompany particular *rpoB* mutations.

Korber's NIH collaborators put the identified *rpoC* mutations into *Mycobacterium smegmatis*, which is closely related to *M. tuberculosis* but easier and safer to work with, and then looked at bacterial growth. They showed that when *rpoB* is mutated for rifampicin resistance, growth declines, but when *rpoC* was also mutated, growth was mostly or completely restored. This showed that compensation was real, but it didn't offer much as to how it worked.

To determine that, Los Alamos structural biologist Chang-Shung Tung made a digital structural model of the *M. tuberculosis* RNA polymerase (RNAP) using RNAPs from other bacteria as a guide. "It wasn't hard to build the structure," he recalls. "The trick is to understand what's going on—how do these two mutations, which are physically far from each other, work together?"

Once again, the answer was in looking at the whole system instead of just one part. The protein structure itself provided few clues, but once Tung added DNA and RNA to the model—RNAP makes RNA molecules using a DNA template and interacts closely with both molecules—the mechanism became clear. It turned out the RNA was the mediator between the two mutations. The *rpoB* mutations causing resistance to rifampicin result in larger, bulkier amino acids being used to build that part of the enzyme, which has the channel-clogging effect associated with fitness loss. But the *rpoC* mutations change the structure and shape of the channel wall, effectively opening the channel from the other end. This gives the RNA molecule a way out and ungums the RNAP's works, letting the bacteria once again reproduce at an unfettered pace.

And there's another thing. With restored fitness and rifampicin resistance, the bacteria are now perfectly poised to take on other antibiotics. Rifampicin is typically administered in a



This 3D model of the RNA polymerase enzyme illustrates the mechanism of compensatory mutations in drug-resistant tuberculosis. The antibiotic rifampicin binds to the polymerase in such a way that nascent RNA (yellow), which is assembled in a special channel on the surface of the enzyme, cannot exit the enzyme complex, so bacterial growth stops. Tuberculosis achieves rifampicin resistance by altering the amino acids at this location (red), thereby changing the polymerase's shape and making the drug unable to bind. But the new shape still partially impedes the enzyme's function, so the resistant bacteria replicate slower than they did before—they have become less fit. Compensatory mutations (green), however, act at the other end of the channel, opening it up and giving the new RNA molecule the room it needs to leave the enzyme complex, thereby restoring fitness to the bacteria. Now they are both resistant and fit.

cocktail of several different drugs, which is very effective for drug-susceptible TB. But if the patient has rifampicin-resistant TB, the drug cocktail won't work as well and could actually increase the likelihood of resistance developing to the other drugs in the cocktail. This is one way patients progress from MDR-TB to XDR-TB.

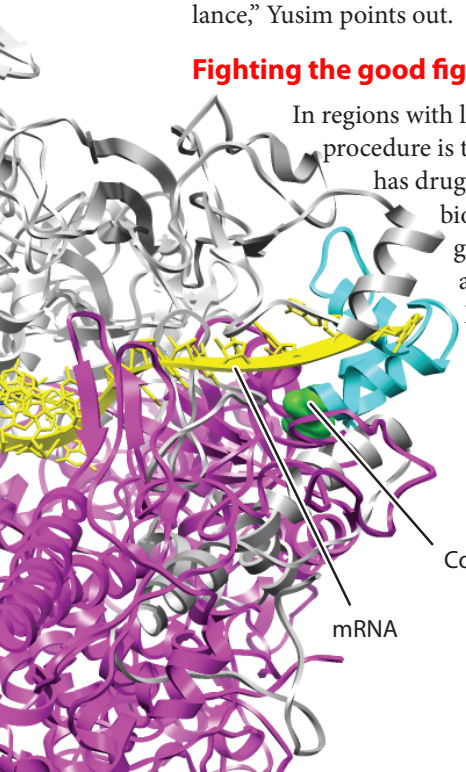
In addition to facilitating resistance to more drugs, compensated drug-resistant forms may also fuel transmission. In the Los Alamos group's analysis of the South Korean data, the same *rpoB-rpoC* mutation pair was found in samples from TB patients who had been treated in the same hospital. That means the resistant, compensated form was transmitted in the hospital. This was the first realization and documentation of compensatory mutations being involved in epidemic spread of TB.

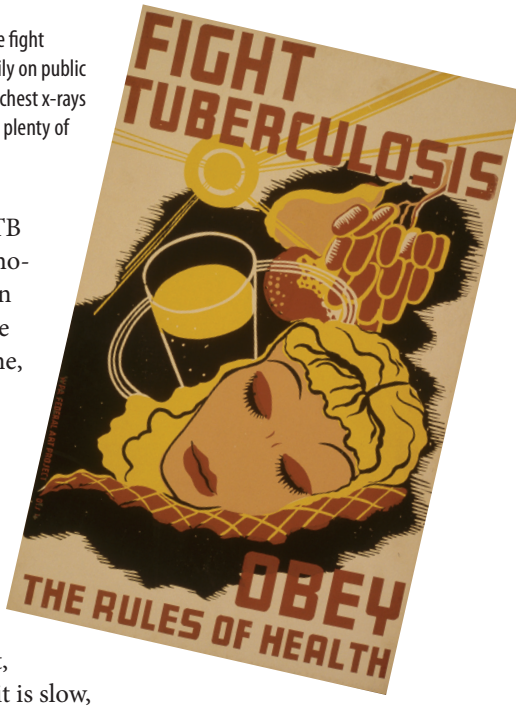
Knowing now that resistant, compensated TB was transmissible, Korber's group next looked at outbreak data from South Africa that had yielded multiple strains of XDR-TB. Revisiting the data revealed that *rpoB-rpoC* resistance and compensatory mutations also underlied these outbreaks. Because the samples didn't have drug-resistance mutations in common for drugs other than rifampicin, upon initial investigation, they were declared to represent independent instances of resistance acquisition. But the Los Alamos team found that all isolates did indeed carry the same *rpoB-rpoC* mutation pair. So resistant and compensated strains were being transmitted in South Africa as well.

The South Korean and South African studies led the group to conclude that compensated rifampicin-resistant strains with improved fitness were being transmitted, leading to subsequent acquisition of different levels of drug resistance in different people. So rifampicin-resistant strains with restored fitness may be fueling the global increase in MDR-TB and XDR-TB cases. "This highlights the importance of studying compensatory mutations and including them in diagnostic screening and epidemiological surveillance," Yusim points out.

Fighting the good fight

In regions with limited resources, the standard procedure is to assume a first-time TB patient has drug-susceptible TB and prescribe antibiotic drugs. But, as the Los Alamos group and others have shown, that assumption is often erroneous. Resistance begets resistance, compensation begets resistance, and both facilitate transmission, making first-time patients who are already resistant more and more common. And TB infections are not homogeneous—a person can be infected by multiple strains at the same time, so just because they coughed up drug-susceptible TB on the day they were tested doesn't mean they aren't dying from XDR-TB. And while testing often isn't done before treatment, even when it is done, it is slow, costly, and not always accurate. Yet skipping the test and going straight to the cocktail for patients who are already infected with resistant TB only compounds the global problem.





Before the age of antibiotics, the fight against tuberculosis relied heavily on public campaigns promoting frequent chest x-rays and healthy lifestyles, including plenty of sun, rest, and nutrition.

So tuberculosis is running rampant, drug-resistance is on the rise, compensation is fueling the fire—what is to be done? There is a commercial kit available, called Xpert MTB/RIF, which is very fast and very reliable and tests for *rpoB* mutations to identify rifampicin-resistant strains. It is backed by the WHO and is getting traction. But it doesn't detect all the possible resistance profiles, let alone the compensatory mutations. (These are both active research areas that are still incompletely defined and too complex for current standard testing.) It only looks at that narrow window in the *rpoB* gene that contains rifampicin-resistance sites. Still, it is a huge step in the right direction.

The infectious disease community has long been aware that emerging drug resistance is a serious problem in the fight against TB. The WHO has made tremendous progress in its massive global campaign to eradicate TB, which focuses heavily on the issue of resistance and early diagnosis. Korber's work, while not offering a quick fix, offers direction in terms of better understanding transmission of drug-resistant TB, the need for rapid diagnostics, and the role of fitness-restoring compensatory mutations.

"What we're saying," she says, "is that the key to global eradication lies in improved and increased clinical testing." More research is needed to develop on-demand multidrug-resistance panels and compensatory mutation tests. Her team's work adds emphasis to that need, bringing humanity one step closer to ending one of its deadliest diseases. LDRD

—Eleanor Hutterer